The EMBO Journal vol.6 no.3 pp.611-616, 1987

# Characterization of recombinant human granulocyte-colony-stimulating factor produced in mouse cells

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Communicated by C.Weissmann

**Mouse C127I cells were transformed with a chimeric plasmid consisting of bovine papillomavirus DNA and human granulocyte-colony-stimulating factor (G-CSF) cDNA placed under the control of the SV40 early promoter. The transformed cells secreted constitutively a high level of human G-CSF, 10-20 μg/ml in a low-serum medium. The secreted G-CSF has been purified to homogeneity by a two-step procedure including gel filtration and hydrophobic column chromatography. The purified recombinant G-CSF runs as a single band with an apparent $M_r$ of 19 000 on a polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. This value corresponds to that of the native human G-CSF purified from the medium conditioned by human carcinoma CHU-2 cells. The recombinant human G-CSF was as active as native G-CSF *in vitro* in supporting proliferation of mouse NFS-60 cells and stimulating colony formation from human as well as mouse bone marrow cells. When the recombinant human G-CSF was subcutaneously administrated into mice, a remarkable stimulation of granulopoiesis and splenomegaly was observed.**

*Key words:* bovine papillomavirus/mouse C127I cells/colony-stimulating factors/hemopoiesis

## Introduction

Colony-stimulating factors (CSFs) are a group of growth factors that support survival, proliferation and differentiation of hemopoietic progenitor cells. CSFs have been classified into at least four types: granulocyte-macrophage CSF (GM-CSF), granulocyte CSF (G-CSF), macrophage CSF (M-CSF) and interleukin 3 (IL-3) (Metcalf, 1985, 1986). Among these CSFs, G-CSF can be distinguished from other CSFs by its ability to stimulate predominantly neutrophilic granulocyte colony formation from bone marrow cells and to induce terminal differentiation of the murine myelomonocytic leukemia cell line WEHI-3B (D+) (Metcalf, 1985, 1986).

G-CSF has been purified to homogeneity from the medium conditioned by lung tissues from mice injected with bacterial endotoxin (Nicola *et al.*, 1983), or human carcinoma cells producing G-CSF constitutively (Welte *et al.*, 1985; Nomura *et al.*, 1986). Both human and mouse G-CSF are glycoproteins (probably *O*-glycosylated), with an apparent $M_r$ of 19 000-24 000 (Nicola *et al.*, 1983; Welte *et al.*, 1985; Nomura *et al.*, 1986). The cDNAs for human and mouse G-CSF have been isolated from human CHU-2 (Nagata *et al.*, 1986a,b), 5637 carcinoma cells (Souza *et al.*, 1986) and murine NFSA fibrosarcoma cells (Tsuchiya *et al.*, 1986), and the chromosomal gene structure for human G-CSF has also been elucidated (Nagata *et al.*, 1986b). Comparison of the structures of human G-CSF cDNAs and the chromosomal gene, revealed that there are two different G-CSF molecules (G-CSFa and G-CSFb) consisting of 177 or 174 amino acids, respectively (Nagata *et al.*, 1986b). They are translated from two different mRNAs generated by an alternative splicing of a single precursor RNA. The cDNAs for human G-CSFs have been expressed in monkey COS cells (Nagata *et al.*, 1986a,b) and *Escherichia coli* (Souza *et al.*, 1986), and the recombinant G-CSFs were active in stimulating colony formation from bone marrow cells and inducing differentiation of murine WEHI-3B (D+) cells or human HL-60 cells.

Here, we have established mammalian cell lines that produce human G-CSF in a large quantity. Mouse C127I cells can be transformed by bovine papillomavirus (BPV), and BPV DNA can be maintained extrachromosomally as multicopy circular DNA (Law *et al.*, 1981). This system was previously used to synthesize human interferons (IFNs) (Fukunaga *et al.*, 1984), and the same strategy was applied to produce human G-CSF. Human recombinant G-CSF produced in mouse C127I cells has been purified to homogeneity and shown to be nearly identical with the native human G-CSF. Furthermore, the *in vivo* effects of this protein on hemopoiesis in normal mice are also described.

## Results

### *Expression of human G-CSF in mouse C127I cells*

Previously, we have established mouse cell lines which constitutively produce human IFNs by using bovine papillomavirus as a vector (Fukunaga *et al.*, 1984). A similar strategy has been applied to produce human G-CSF in mammalian cells, and the scheme for the construction of human G-CSF expression plasmid is shown in Figure 1. Since the apparent specific activity of human G-CSFb protein was >10 times higher than that of human G-CSFa protein (Nagata *et al.*, 1986b) the G-CSFb cDNA was first expressed. The human G-CSFb cDNA excluding the 3′ non-coding sequence was inserted at the *Bam*HI site of the pdKCR vector (O'Hare *et al.*, 1981; Nagata *et al.*, 1986b) and the *Eco*RI site was changed to a *Hin*dIII site. The resultant plasmid (HinpHGV21) contains human G-CSF cDNA immediately downstream of the SV40 early promoter and upstream of the rabbit β-globin intron splice sites and the SV40 polyadenylation site. When this plasmid DNA was introduced into monkey COS cells, G-CSF activity could be detected transiently in the medium of COS cells 72 h after transfection (Nagata *et al.*, 1986b). To establish the cell line producing G-CSF constitutively, the hybrid gene consisting of the SV40 early promoter, human G-CSF cDNA and rabbit β-globin splice sites, was joined with the 8.4 kb *Hin*dIII-*Pvu*II fragment of pdBPV-1 to yield pTNV2. The 8.4-kb *Hin*dIII-*Pvu*II fragment of pdBPV-1 contains the 5.4 kb of *Hin*dIII-*Bam*HI fragment of BPV DNA which is necessary for transformation of mouse C127I cells and maintenance of BPV DNA as extrachromosomal DNA in mouse cell (Law *et al.*, 1981; Sarver *et al.*, 1982).

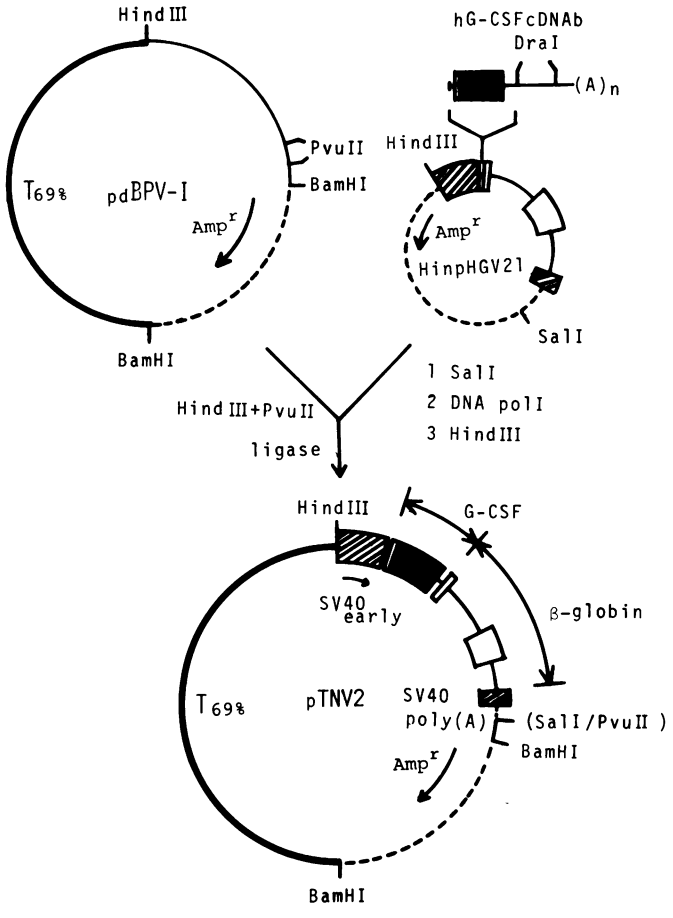


**Fig. 1.** Construction of human G-CSF expression plasmid. The hatched boxes represent the sequences of SV40 early promoter and polyadenylation signal. The closed box on the human G-CSF cDNA indicates the coding sequence for mature protein while the open box indicates that for signal peptide. On the β-globin gene, two open boxes and the line between them represent the truncated second exon, third exon and the second intron of the rabbit β-globin gene. The *Hin*dIII−*Bam*HI fragment of pdBPV-1 ($T_{69}$) necessary for transformation is indicated by the heavy line. The dashed lines are bacterial DNA sequence derived from pML-2. The restriction sites used for the plasmid construction are indicated. Amp$^r$, β-lactamase gene.

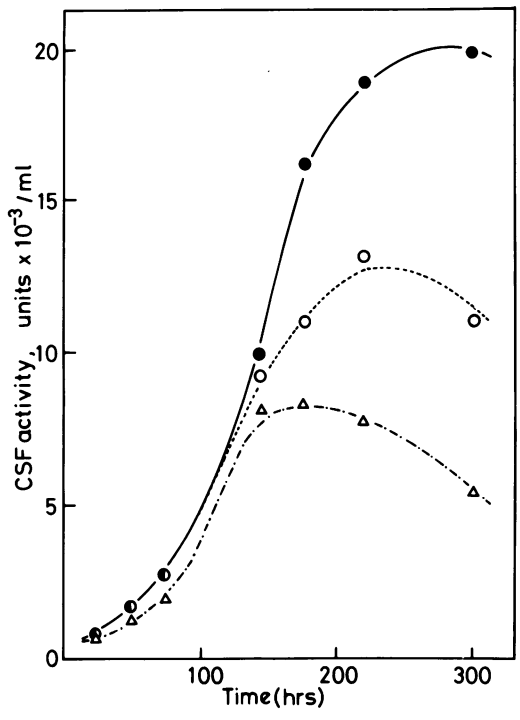


**Fig. 2.** Effect of the serum concentration on G-CSF production by the transformed V216 cells. V216 cells were seeded at $3 \times 10^4$ cells per 3.5-cm plate, and grown to apparent confluency in DMEM containing 5% FCS. Then cells were fed with fresh medium (time 0) containing 5% (●), 1% (○) or 0.1% (Δ) FCS, and cultured for 12 days. Aliquots of the medium were withdrawn at the indicated time, and assayed for G-CSF activity by using murine NFS-60 cells as described in Materials and methods.

Prior to transfection, the plasmid pTNV2 was digested with *Bam*HI to remove the bacterial sequence of pML-2 that might be inhibitory for transformation. Transfection of the DNA into mouse C127I cells was carried out by the Ca-phosphate co-precipitation method (Wigler *et al.*, 1978), and morphologically altered macroscopic foci could be observed 18 days after transfection. The number of foci was 30 with 20 μg of pTNV2 plasmid while 843 foci were obtained with 20 μg of the intact linear viral DNA (*Bam*HI-digested pdBPV-1). Such a reduction of the transformation efficiency with the hybrid plasmid was also observed previously with the BPV-IFN expression plasmid (Fukunaga *et al.*, 1984).

A total of 30 foci transformed with pTNV2 and five foci with pdBPV-1 was picked up and expanded in DMEM containing 5% FCS. Since the transformed cells can grow faster than the non-transformed cells (Fukunaga *et al.*, 1984), the cells were passaged three times to reduce the non-transformed C127I cells contaminated in the transformed cells. After the last passage, the cells were grown in the fresh medium for 5 days, and the CSF activity in the medium was assayed by using mouse NFS-60 cells as described in Materials and methods. All cell lines transformed with the pTNV2 plasmid could produce G-CSF although the G-CSF activities differed among these cell lines. On the other hand, none of the cell lines obtained by transformation with pdBPV-1 produced a detectable level of G-CSF.

*Properties of the transformed mouse C127I cells producing G-CSF*

The C127I cells transformed with BPV exhibit an array of properties related to cellular transformation including the growth in a low serum medium (Law *et al.*, 1978; Sarver *et al.*, 1982). To purify G-CSF from the medium conditioned by the pTNV2 transformed cells, we first studied the effect of serum concentrations on the production of G-CSF. The cell line V216, which has produced the highest level of G-CSF, was grown to confluency in DMEM containing 5% FCS. The medium was changed to DMEM containing either 5, 1 or 0.1% FCS, and at the indicated time, the G-CSF activities were measured by using aliquots of the culture supernatant. As shown in Figure 2, in the presence of 5 or 1% FCS, G-CSF activity in the culture medium increased exponentially, and reached to $1.8 \times 10^4$ units/ml or $1.3 \times 10^4$ units/ml, respectively, at day 9. On the other hand, in the presence of 0.1% FCS, the level of G-CSF began to decresae at day 5 after reaching $7.5 \times 10^3$ units/ml and the cells detouched from the dish. Since the homogeneous native G-CSF protein, purified from the medium conditioned with CHU-2 cells, has the specific activity of $8.5 \times 10^6$ units/mg protein (Table I) these results suggest that the cell line V216 can produce human G-CSF up to 10−20 μg protein/ml of the medium. When the medium containing ~4000 units of G-CSF activity was directly analysed on a polyacrylamide gel electrophoresis, a band of $M_r$

**Table I.** Summary of purification of the recombinant human G-CSF

| Steps | Protein (mg) | Activity (units × $10^6$) | Specific activity (units × $10^6$/mg) |
|---|---|---|---|
| Supernatant of V216[a] | 600 | 144 | 0.24 |
| AcA54 Ultrogel | 30 | 138 | 4.6 |
| Butyl-Toyopearl | 7.9 | 72 | 9.1 |
| Native human G-CSF[b] | | | 8.5 |

[a]Obtained from 1.2 l of medium conditioned by the cell line V216.
[b]Homogeneous human G-CSF purified from the medium conditioned by human carcinoma CHU-2 cells (Nomura *et al.*, 1986).

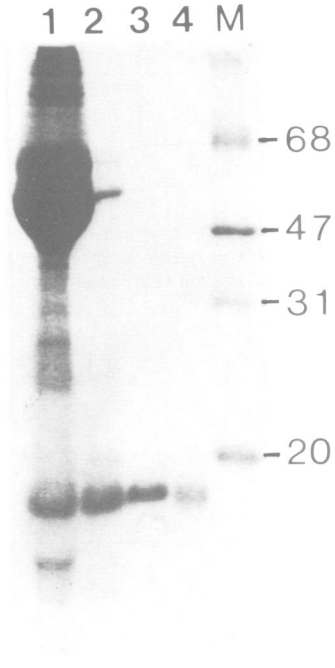


**Fig. 3.** SDS/polyacrylamide gel electrophoresis of the purified human G-CSF. Protein samples from each purification step were heated at 90°C for 3 min without reducing agents and analysed on a 12.5% polyacrylamide gel in the presence of 0.1% SDS. **Lane 1**, medium conditioned by the cell line V216 (20 μg); **lane 2**, pooled fractions from AcA 54 column (2.0 μg); **lane 3**, the purified recombinant G-CSF after Butyl-Toyopearl column (1.0 μg); **lane 4**, native G-CSF purified from the conditioned medium of CHU-2 cells (Nomura *et al.*, 1986) (0.5 μg); **lane M**, mol. wt markers. Sizes of marker proteins are given in kds.

19 000 corresponding to that of the native G-CSF was observed (Figure 3, lanes 1 and 4).

The cell line V216 could be maintained without passage for at least 2 months in DMEM containing 1% FCS by changing the medium every 4 days, and the rate of G-CSF production did not change during this period.

*Purification of the recombinant G-CSF from the medium conditioned by V216 cells*

Purification of the recombinant human G-CSF was started from 1200 ml of the medium conditioned by the cell line V216. Since mol. wts of the major contaminated proteins were >50 000 (Figure 3, lane 1) the medium was concentrated ~100 times and chromatographed on an Ultrogel AcA 54 column. The G-CSF activity was eluted as a single peak at the position corresponding to the apparent $M_r$ of 24 000, and could be separated from most of the contaminated proteins, yielding about 19-fold purification (Table I and Figure 3, lane 2). The active fractions of the AcA 54 gel filtration column were combined and applied to a Butyl-Tyopearl column attached to an f.p.l.c. system (Pharmacia). The G-CSF protein was eluted from the column by decreasing the concentration of $(NH_4)_2SO_4$, yielding a further 2-fold purification. The overall purification by this simple two-step procedure was about 38-fold with a yield of 50% (Table I).

When the purified recombinant G-CSF was analysed on polyacrylamide gel electrophoresis in the presence of SDS, a single band of $M_r$ 19 000 was observed (Figure 3, lane 3) which corresponds to that of the native human G-CSF purified from the medium conditioned by human carcinoma CHU-2 cells (Figure 3, lane 4) (Nomura *et al.*, 1986). The $NH_2$-terminal amino acid sequence of the recombinant human G-CSF was Thr-Pro-Leu-Gly- - - (N.Kubota and H.Nomura, unpublished), which agreed with that of the native human G-CSF (Nomura *et al.*, 1986). This result indicates that the murine C127I cells transformed with pTNV2 can cleave the signal sequence of the human G-CSF precursor protein correctly as human CHU-2 carcinoma cells. Furthermore, the sugar moiety detected in the preparation of the homogeneous recombinant G-CSF was 1 Gal, 1 GalNAc and 2 NeuNAc per molecule (M.Oheda and H.Nomura, unpublished), while the native G-CSF protein had 1 Gal, 1 GalNAc and 1.5 NeuNAc per molecule (Nomura *et al.*, 1986), suggesting a similar glycosylation has occurred on the human G-CSF molecule produced in murine C127I cells and in human CHU-2 carcinoma cells.

*In vitro biological activity of recombinant G-CSF*

Human G-CSF and mouse G-CSF support proliferation of mouse myeloid leukemia NFS-60 cells (Weinstein *et al.*, 1986) which were originally isolated as IL-3-dependent leukemia cells (Holmes *et al.*, 1985). And the stimulation of [$^3$H]thymidine incorporation into NFS-60 cells was used to assay the G-CSF activity during purification of the recombinant human G-CSF (Table I). The concentration of G-CSF necessary to give a half-maximal stimulation was $\sim 5 \times 10^{-11}$ M by either the native or the recombinant homogeneous G-CSF (Table I). On the other hand, the other IL-3-dependent cell lines such as murine FDC-P1 (Dexter *et al.*, 1980) or murine IC-2 cells (Koyasu *et al.*, 1986) did not respond to the native or the recombinant G-CSF up to 1 μM of G-CSF protein (data not shown).

Colony-stimulating activities of the native and the recombinant G-CSF were then examined with human and mouse bone marrow cells. As shown in Figure 4, the dose-response curves with the recombinant G-CSF were very similar to those with the native G-CSF by using either human or mouse bone marrow cells as target cells. The half maximal response was obtained with 3.5 ng G-CSF/ml for human bone marrow cells and with 2.5 ng G-CSF/ml for mouse bone marrow cells. These results indicate that the recombinant human G-CSF produced by mouse C127I cells is as active as the native human G-CSF *in vitro*, and human G-CSF can work on mouse as well as on human progenitor cells.

*In vivo studies of G-CSF*

To investigate the role of G-CSF in regulating haemopoiesis *in vivo*, 5 μg of the recombinant human G-CSF was injected daily subcutaneously into mice, and changes in the peripheral blood cell counts were examined. As shown in Figure 5A, the number of granulocytes was found to increase 12 h after the first injection of G-CSF, and thereafter, five to six times more granulocytes than the basal level were maintained during the administration of G-CSF. When the injection of G-CSF has ceased the number returned to the basal level within 48 h. On the other hand, the

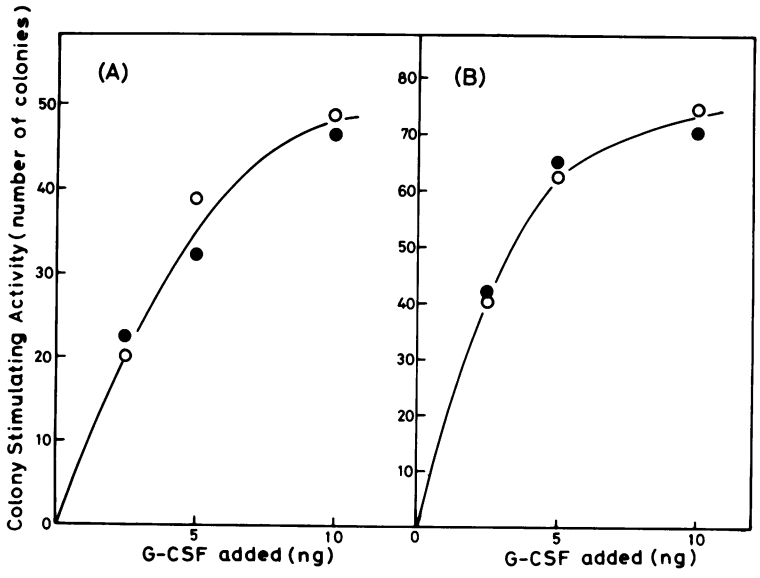


**Fig. 4.** Colony-stimulating activity of human G-CSF *in vitro*. The indicated amounts of the human recombinant (○) or the native (●) G-CSF were assayed for colony-stimulating activity by using human **(A)** or mouse **(B)** bone marrow cells. In the human system, no colony consisting of more than 20 cells was formed without G-CSF, while in the mouse system, seven colonies were observed without G-CSF and subtracted from each value.

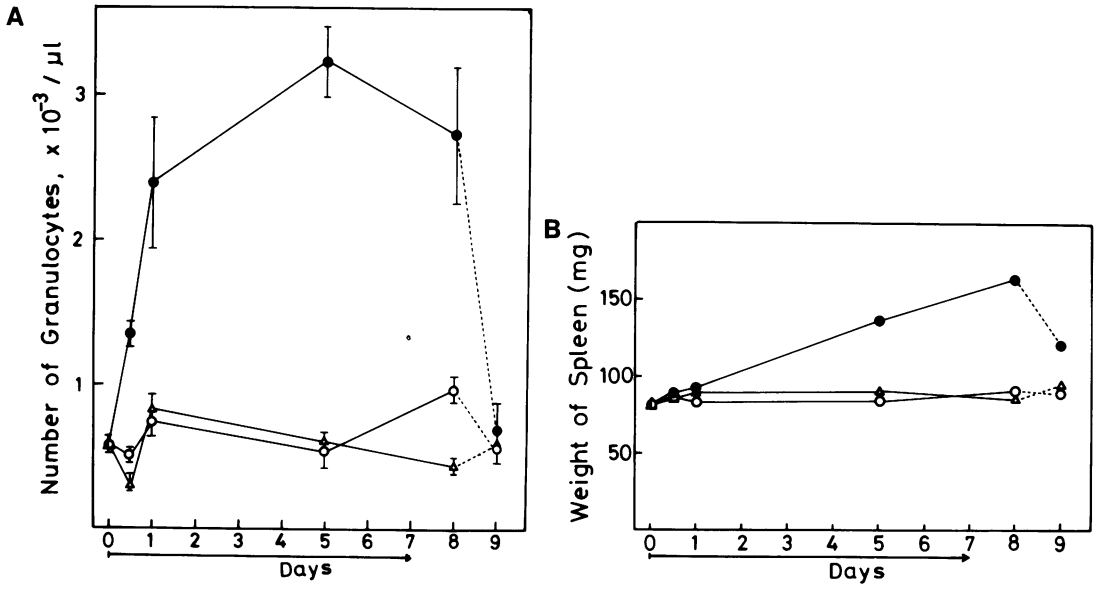


**Fig. 5.** *In vivo* effects of the human recombinant G-CSF in mice. Mice were injected daily subcutaneously with G-CSF (●), LPS (○) or saline containing 10% mouse serum (Δ) for 8 days (day 0 to day 7). At the indicated day, four mice of each group were killed for blood and spleen examinations. **(A)** The number of neutrophilic granulocytes in peripheral blood. Each point represents the mean value obtained from four mice ± standard error. **(B)** The weight of spleen. Each point is the averge weight of the spleen from four mice.

numbers of monocytes, lymphocytes, platelets and erythrocytes in the peripheral blood did not increase significantly by the administration of G-CSF (data not shown).

Consistent with the change of granulocyte number, the weight of spleen has gradually increased during this period (Figure 5B) and at day 8 it has reached 160 mg which is about twice as much as that of normal spleen (80 mg). Morphological analysis of the spleens revealed that the increase in the weight was mostly due to the stimulated production of neutrophilic granulocytes. The granulocytosis and splenomegaly caused by G-CSF was dose-dependent (data not shown), whereas no change of the granulocyte number and the spleen weight was observed in the control mice which received saline or *E. coli* LPS (Figure 5).

## Discussion

Souza *et al.* (1986) have expressed human G-CSF in *E. coli* to produce a large amount of the protein. Although the non-glycosylated G-CSF protein produced in *E. coli* is active *in vitro* (Souza *et al.*, 1986), it is still desirable to carry out the study with the correctly processed G-CSF molecule. This is because the foreign protein synthesized in *E. coli* is not only non-glycosylated, but also often biochemically different from native proteins at the $NH_2$- or COOH-terminus (Rose *et al.*, 1983) or antigenetically (Le *et al.*, 1984).

The mouse cell line established in this report could produce human G-CSF constitutively up to 10–20 µg protein per ml of the

culture medium (Figure 2), which is at least 100 times higher than the value obtained with human carcinoma cells, CHU-2 (Nomura *et al.*, 1986) or 5637 (Welte *et al.*, 1985), and probably corresponds to that obtained in *E. coli* harbouring the CSF gene (Souza *et al.*, 1986). The rate of production of human G-CSF by the transformed mouse cells was nearly 10 times higher than that of human IFNs (Fukunaga *et al.*, 1984), although the same expression system was applied to both genes. It may be explained by the fact that IFNs but not G-CSF have cytotoxic activity, and only cell lines producing low levels of IFNs were selected during propagation of the transformed mouse cells.

The mouse C127I cells transformed with the BPV-G-CSF expression plasmid could secrete G-CSF in the low-serum medium, and the purification of G-CSF by the simple two-step procedure could yield a homogeneous preparation of G-CSF (Figure 3, Table I). The native human G-CSF molecule produced by human CHU-2 cells is probably a mixture of G-CSFa and G-CSFb proteins consisting of 177 and 174 amino acids, respectively (Nagata *et al.*, 1986b), while the recombinant G-CSF expressed in this report is G-CSFb. The specific activities of G-CSF protein in stimulating proliferation of murine NFS-60 cells and colony formation from bone marrow cells were nearly identical between the native G-CSF and the recombinant G-CSFb. This result was expected since >80% of G-CSF mRNA found in CHU-2 cells is G-CSFb mRNA (Nagata *et al.*, 1986b). Recently, the G-CSFa cDNA was also expressed in mouse C127I cells using a similar BPV-G-CSF expression plasmid (M.Tsuchiya and S.Nagata, unpublished), purification and characterization of the recombinant G-CSFa molecule may reveal the functional differences between G-CSFa and G-CSFb proteins.

The subcutaneous administration of the recombinant G-CSF into mice caused a remarkable stimulation of granulopoiesis associated with splenomegaly (Figure 5). In contrast to observations with murine IL-3 (Kindler *et al.*, 1986; Metcalf *et al.*, 1986) or human GM-CSF (Donahue *et al.*, 1986), no significant change was observed in the number of monocytes, lymphocytes and erythrocytes in the peripheral blood. These results are consistent with the observations (Asano *et al.*, 1977; Bessho *et al.*, 1984) that granulocytosis and splenomegaly were developed in nude mice which had been transplanted with tumour cells producing CSF, and demonstrated that G-CSF is a potent stimulator for granulopoiesis *in vivo*. There is a possibility that the continued release of G-CSF from certain cells into blood may be responsible for the granulocytosis associated with certain infections. Actually, some G-CSF activity could be detected in human sera of patients who suffered from bacterial infection (N.Shirafuji, S.Asano and S.Nagata, unpublished). However, these results do not necessarily mean that the production of granulocytes in normal haemopoiesis is also regulated by G-CSF circulating in the blood. This may be acomplished by a local release of G-CSF from stromal cells associated with haemopoietic cells, or cell-to-cell interaction in bone marrow or lymphoid organs (Whetton and Dexter, 1986). To examine the mechanism of the production of granulocytes *in vivo*, it will be necessary to identify the G-CSF-producing cells in the process of normal haemopoiesis and granulocytosis.

The demonstration that G-CSF is a potent stimulator for granulocytosis in mice provides a model system for testing potential clinical uses of the protein. For example, when G-CSF was injected into mice which had developed granulocytopenia by treatment with cyclophosphamide, the duration of granulocytopenia was shortened (M.Tamura, K.Hattori, H.Nomura, M.Oheda, N.Kubota, I.Imazeki, M.Ono, Y.Ueyama, S.Nagata, N.Shirafuji and S.Asano, in preparation), which possibly reduced the risk of severe infection. These experiments may prove valuable for G-CSF therapy in the treatment of patients undergoing bone marrow transplantation and cancer therapy.

## Materials and methods

*Construction of the G-CSF expression plasmid*

Construction of the BPV-G-CSF hybrid plasmid is shown in Figure 1. Plasmid HinpHGV21 was derived from pHGV21 (Nagata *et al.*, 1986b) by changing one of two *Eco*RI sites to a *Hin*dIII site using a *Hin*dIII linker. On the HinpHGV21 plasmid, a 719-bp human G-CSFb cDNA (*Eco*RI−*Dra*I fragment) was placed under control of the SV40 early promoter. HinpHGV21 was digested with *Sal*I (on pBR322 sequence) and the end was blunted with DNA polymerase I (Klenow fragment). After cleavage with *Hin*dIII, the large DNA fragment (2.9 kb) containing the G-CSF cDNA was purified on an agarose gel (Low Gel Temperature, Bio-Rad), and ligated to the 8.4-kb *Hin*dIII−*Pvu*II fragment of pdBPV-1 (Sarver *et al.*, 1982) to yield pTNV2.

*Transformation of mouse C127I cells*

Transfection of mouse C127I cells (ATCC, CRL1616) with pTNV2 was carried out as described previously (Fukunaga *et al.*, 1984). The morphologically altered macroscopic foci were counted 18 days after transfection. Individual foci were isolated by using cloning cylinders, and grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Seiyaku, Tokyo) containing 5% fetal calf serum (FCS, Gibco).

*Purification of human G-CSF*

One of the transformed cell lines (V216, see Results) was seeded at $3\times10^5$ cells per 10-cm plate, and grown in DMEM containing 5% FCS. When the culture became confluent, the medium was switched to DMEM containing 1% FCS, and by changing the medium every 4 days, a total of 1.2 l of the conditioned medium was collected. The medium was concentrated to ~100 ml using a Diaflow PM 10 membrane (Amicon) and further to 10 ml by dialysing against 50% (w/v) polyethylene glycol 6000 in phosphate-buffered saline ($P_i$/NaCl). Aliquots (5 ml) of the concentrated sample were chromatographed on an Ultrogel AcA 54 column (LKB, 2.7 × 70 cm) equilibrated with 10 mM Tris−HCl buffer (pH 7.5) containing 150 mM NaCl and 0.02% Tween 20. The active fractions were combined and concentrated with a Diaflow PM-10 membrane. The solution was adjusted to 0.7 M $(NH_4)_2SO_4$ and applied on a Butyl-Toyopearl column (1.5 × 10cm, Toyo Soda Co., Tokyo) which was equilibrated with 0.1 M sodium phosphate buffer (pH 6.0) containing 0.64 M $(NH_4)_2SO_4$ and 0.02% Tween 20. G-CSF was eluted with a linear gradient consisting of 20 ml of the equilibration buffer and 20 ml of 0.02% Tween 20. The fractions containing G-CSF activity were combined and concentrated over a PM-10 membrane in a stirred cell, and dialysed against $P_i$/NaCl.

*Biological assay of G-CSF and determination of protein concentration*

G-CSF activity was assayed by stimulation of [$^3$H]thymidine incorporation into the factor-dependent mouse NFS 60 myeloid leukemia cells as described previously (Tsuchiya *et al.*, 1986). One unit of the activity represents the concentration of G-CSF which gives a half-maximal stimulation with $5\times10^4$ cells per 100 μl. The assay procedure for *in vitro* colony-stimulating activity with human or mouse bone marrow cells was described previously (Nagata *et al.*, 1986a; Nomura *et al.*, 1986). The protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard.

*Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis in the presence of SDS was carried out as described (Laemmli, 1970), and protein was detected by staining with 0.2% Coomassie Brilliant Blue. As a size marker a mixture of proteins, consisting of bovine serum albumin ($M_r$ 68 000), EF-Tu from *E. coli* ($M_r$ 47 000), DNase I from bovine pancreas ($M_r$ 31 000) and soybean trypsin inhibitor ($M_r$ 20 100), was electrophoresed in parallel.

*In vivo studies*

The homogeneous human recombinant G-CSF was dissolved in 0.15 M NaCl containing 10% mouse serum (prepared from Balb/c mice) at the concentration of 50 μg per ml. As controls, solutions without G-CSF or with *E. coli* lipopolysaccharide (LPS, Sigma, 4.0 ng/ml) instead of G-CSF were also prepared. Each sample (0.1 ml/mouse) was injected daily into the subcutaneous space of 8−10-week-old male Balb/c mice which were bred in specific pathogen-free conditions. At the indicated time, four mice in each group were bled from the retro-orbital plexus and killed for haematological examinations. The number of blood cells was counted using a hematocytometer, and blood smear and spleen cells stamps were stained with May-Giemsa solution for differential counts.

## Acknowledgements

We thank Drs N.Shirafuji and H.Kodo for their help during *in vivo* experiments. Thanks are also due to Dr T.Yamazaki for his encouragement and discussions. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

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*Received on November 26, 1986*